Adsorption of Bovine Serum Albumin onto Hydroxylapatite: Theoretical Modeling and Measurements

Modarress, Hamid*+

Faculty of Chemical Engineering, Amirkabir University of Technology, Tehran, I.R. IRAN

Mohsen-Nia, Mohsen

Faculty of Chemical Engineering, University of Kashan, Kashan, I.R. IRAN

Allafkari, Leila

Faculty of Chemical Engineering, Amirkabir University of Technology, Tehran, I.R. IRAN

ABSTRACT: *Adsorption of Bovine Serum Albumin (BSA) onto calcium Hydroxyl Apatite (HA), $Ca_{10}PO_4(OH)_2$, has been studied by Ultra Violet (UV) spectroscopy. The adsorption isotherms of BSA onto the HA surface at 291.7 K and 303.2 K were satisfactorily presented by Langmuir equation and the evaluated parameters are reported. The specific surface area of HA has been measured by the BET method. The obtained value for the used HA sample was 63 m^2/g. The effect of pH on BSA adsorption onto the HA in presence of salts KCl and NaF and phosphate ion has been investigated. In the fixed concentration of BSA (1 mg/mL) it is shown that the pH decreasing causes to increase of the adsorption of BSA onto HA. Considering the role of effective factors in BSA adsorption onto HA such as the size and charge of the BSA and the surface energy of HA showed that the electrostatic repulsion forces between HA and BSA cause a decrease in BSA adsorption onto HA. Also the obtained results indicated that the surface energy of HA predominates the effect of size and charge of BSA.*

KEY WORDS: *Adsorption isotherm, Hydroxylapatite, BSA, Langmuir, BET.*

INTRODUCTION

Hydroxylapatit (HA), $[Ca_{10}(PO_4)_6(OH)_2]$ which is particular bioactive ceramic can be transformed to biological apatite through a set of reactions including dissolution, precipitation, and ion exchange [1, 2].

Studying on the interaction between protein and calcium phosphate implants is one of the basic subjects in biomaterials science and engineering [3-5]. The calcium phosphate ceramics have good biocompatibility, bioactivity and osteoinduction under certain conditions. The initial events occurring upon implantation of biomaterials is the spontaneous adsorption of monolayer of proteins coming from physiological fluids, which could play

* *To whom correspondence should be addressed.*

+ *E-mail: hmodares@aut.ac.ir*

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a vital role in determining the nature of the tissue implant interface and be related to the good biological behavior of the calcium phosphate ceramics. Thus the interfacial interactions between protein molecules and the implant surface have been extensively investigated in the recent years. Protein adsorption is a complex process and influenced by properties of both protein and implants surface [1, 6, 7].

HA is the principal inorganic component of human bone matrix. The major subphase of the mineral consists of submicroscopic crystals of an apatite of calcium and phosphate, resembling crystal structure of $[Ca_{10}(PO_4)_6(OH)_2]$ [10]. HA has recently attracted a great deal of attention because of high affinity for biopolymers such as proteins and enzymes together with its excellent biocompatibility and non-toxicity. Many studies have employed HA with in High Performance Liquid Chromatography (HPLC) for separating biopolymers [11]. Moreover HA is used as implant materials for replacement and repair of damaged or diseased bone and as plasma-sprayed coating for metal prosthesis used in knee and hip replacement. It is observed that the host response to HA is favourable [10].

When HA ceramics are implanted into the body, a number of events occur which affect subsequent cell attachment. This can include apatite layer formation by dissolution and precipitation of the ceramics, precipitation solely from the surrounding liquid and protein adsorption. Protein becomes adsorbed to the substrate surface from the surrounding body fluid, seconds or minute after immersion [11]. Studies on protein interactions at interfaces have been common since the early part of the century, and these studies have provided much of the information in this field [12-14].

HA is stable *in vivo* condition and has been used in hard tissue repair applications, such as implant coating [15] and bone substitutes [16]. The mineral component of bone and teeth consists highly substituted HA in crystalline or amorphous forms. However, the common mineral impurities in biominerals such as sodium, potassium and magnesium components [17] may affect the behavior of HA when it is used as an implant in human body. Although there are a number of studies on adsorption of protein onto HA [3, 6, 18], there is still need for theoretical and experimental studies on protein adsorption onto HA in presence of salts to elucidate its behavior in human body.

HA is in the space group $P6_3/m$ and its unit cell dimensions are a = b = 0.943 nm and c = 0.688 nm possessing two different binding sites in the crystal surface (**C** and **P**) [19, 20]. The **C** sites are arranged on ac or bc crystal faces in a rectangular manner, with the interdistance in the a or b direction equal to 0.943 nm and the interdistance in the c direction equal to 0.344 nm (c/2). The **P** sites are arranged hexagonally on the ab crystal face, with a minimal distance of 0.943 nm. The **C** sites are rich in calcium ions or positive charge and thus bind to acidic groups of proteins, but the **P** sites lack calcium ions or positive charge and therefore attach to basic groups of proteins [21].

Proteins are long chains of amino acid [22]. Some of these amino acids carry side chain carboxyl or amino groups, and these may remain free and exposed to the solvent when the protein is in solution. Therefore, they can dissociate in aqueous solution at a suitable pH, resulting in $-COO^-$ and $-NH_3^+$ ions covalently attached to the protein macromolecule [23-25]. The carboxyl group tends to ionize at pH values over about 4 and the amino group at below about 12. Thus, in acid solution, a typical protein becomes positively charged because of the presence of $-NH_3^+$ and $-COO^-$ groups, and in basic solution it is charged negatively because of $-NH_2$ and $-COO^-$ groups. When pH equals pI, the net charge is zero. This corresponds to the presence of equal numbers of oppositely charged groups on the protein. At pH's near the pI , both $-NH_3^+$ and $-COO^-$ groups are present, so that the net charge is small [24-26]. In this way, proteins have multiple- site binding which often results in irreversible adsorption. By these means, HA contains multiple binding sites, and the proteins have multiple groups with affinity for the HA sites, cooperatives adsorption results [26]. Among all the proteins, BSA is most common and deeply studied [27-29].

Due to lack of information about adsorption capacity of BSA onto HA, and the factors affecting the adsorption such as pH and temperature especially in presence of salts, in this work the adsorption behavior of BSA onto the HA surface is investigated at varied pHs and temperatures in presence of salts KCl, NaF and KH_2PO_4. The concentration of BSA in solutions in contact with HA surface were measured by UV spectroscopy.

EXPERIMENTAL SECTION

Materials and Equipments

Bovine serum albumin (Albumin Fraction V) and other chemicals of analytical grade used in the present study were purchased from Merck Co. Inc., Germany and used without further purification. The specific surface area of the used commercial grade HA was determined by a surface area analyzer (BET) Autosorb-1MP, Quantachrome, USA. Measurement of protein concentration was carried out with UV spectrophotometer (Cintra5).

Specific surface area measurements

BET theory is a well-known rule for the physical adsorption of gas molecules on a solid surface [30]. The concept of the theory is an extension of the Langmuir theory, which is a theory for monolayer molecular adsorption, to multilayer adsorption. The resulting BET equation is expressed in the Appendix A.

In the BET measurements the adsorbate was liquid N_2 at 77 K with an adsorption cross section s = 16.2 $Å^2$ and the adsorbent was HA. The operating condition for gas sorption instrument is given in Table 1. The specific surface area of the HA sample was determined from the plot of 1 / v [(P_0 / P) − 1] versus P / P_0 according to Eqs. (A-3) and (A-4) as presented in Fig. 1. The specific surface area was determined from the slope and the intercept of this plot. The value of specific surface area for the HA sample was obtained as 63 m^2/g. The surface area of HA depends on synthetic and heat treatment methods used to obtain a varied crystalline structures. The specific surface area of synthesized HA are normally in the range of 30-100 m^2/g [24, 31]. Therefore the measured specific surface areas for the HA used in this study is in the normal range, depending on its crystalline structure.

Protein adsorption measurements

The aqueous solutions containing BSA, salts were made by mass using a Sartorius analytical balance (Model A200S with an uncertainty ±0.0001g). BSA, solutions (0.5-2.5 mg/mL) were prepared in 0.002 M sodium acetate-acetic acid buffer (pH range 3.7-5.6) and in KH_2PO_4/NaOH buffer (pH range 5.8-8.0). To study adsorption of BSA from prepared solutions onto the solid HA surface, the solid and liquid phases were let to contact in a laboratory made sealed contact device tube of 10 mL volume. For each experimental adsorption measurement, the amount of 0.1 g HA and 5 mL of prepared protein solutions were added to the contact device tube and was shaken for 30 minutes in a water thermostatic bath (±0.1◦C uncertainty) and then was let to settle for 30 minutes to achieve equilibrium. After that, the solutions were centrifuged for 10 minutes. Finally, the supernatant was collected and detection of BSA concentration was performed by UV spectroscopy measurements at 280 nm, according to the calibration curve previously made. In another experimental work, BSA solutions were prepared (0.5 mg/mL) with addition of the salts to the solutions. The salts and the concentration range were: KCl (0.005-0.10) M; NaF (0.005-0.025) mg/mL; K_2HPO_4 (0.05-0.2) M.

The experimental measurements were repeated at least three times and the average was taken as the adsorbed amount of BSA. The reproducibility of the measured concentrations was found to be within ±0.15%, and the maximum deviations from the average value were always <0.1%.

Table 1: Operating condition for gas sorption instrument.

Adsorbate	Nitrogen
Cross-Section Area	16.2 Å/molecule
Sample weight	0.033 g
Outgas Temperature	373.2 K
Outgas time	12 min
Batch Temperature	350.5 K
Analysis Time	57.5 min

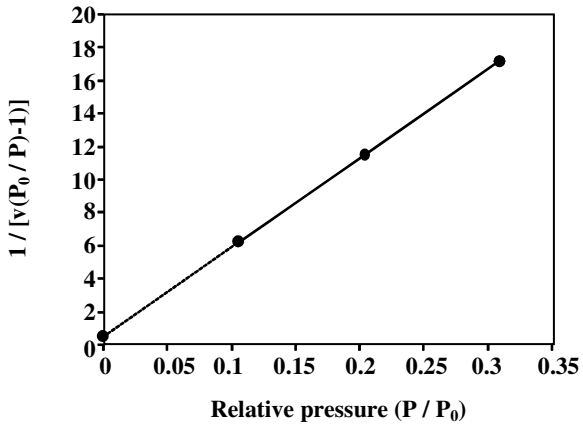

Fig. 1: BET plot for measuring specific surface area of HA.

RESULTS AND DISCUSSION

Fig. 2 shows the effect of temperature on adsorption capacity (mg/m^2) of BSA onto HA. It is shown that increasing temperature slightly increases the adsorption capacity of BSA onto HA.

It can be attributed to the increase in thermal activity of BSA which causes higher molecular diffusion of BSA toward the surface of absorbent HA [32]. The adsorption isotherms of BSA onto the HA surface at 291.7 K and 303.2 K were satisfactorily presented by Langmuir equation as a most commonly used equation to describe adsorption phenomena in various systems [18,33]:

$$\frac{1}{q} = \frac{1}{q_s K_a C} + \frac{1}{q_s} \quad (1)$$

Where, q and q_s are respectively equilibrium and maximum concentrations (mg/unit adsorbent) of adsorbed solute, C is equilibrium solution concentration of solute (mg / unit solution) and K_a is Langmuir equilibrium constant.

The assumptions involved in Langmuir isotherms indicate that one molecule adsorbs on one site of adsorbent and there are no lateral interactions between adsorbed solute molecules. Fig. 3 shows the linear plot of 1/q versus 1/C for adsorption of BSA onto HA surface at 291.7 K and 303.2 K. According to this figure the maximum adsorbed BSA on HA surface are 70.42 mg/m^2 and 81.96 mg/m^2 respectively at 297.1 K and 303.2 K.

The experimental results for adsorption of BSA onto HA are correlated by using Langmuir isotherm equation, Eq. (1), and the evaluated parameters and square of the correlation coefficients (R^2) are presented in Table 2. The calculated R^2 values indicate that the experimental isotherms data favourably correlated by the Langmuir model.

The obtained isotherm data are also correlated by Freundlich isotherm equation. The Freundlich equation is expressed as [34]:

$$q_e = K_f C_e^{1/n} \quad (2)$$

where K_f and n are Freundlich constants. To determine the constants K_f and n, the linear form of the Eq. (2) can be considered in the following form:

$$\ln q_e = \ln K_f + \frac{1}{n} \ln C_e \quad (3)$$

Table 2. Langmuir parameters calculated, Eq.(1), from experimental results for adsorption of BSA onto HA at different pHs.

pH	K_a	q_s
3.8	1.21	24.01
4.6	1.11	19.98
5.0	0.95	15.10
5.6	0.90	12.51
7.2	0.88	7.72
8.0	0.70	3.31

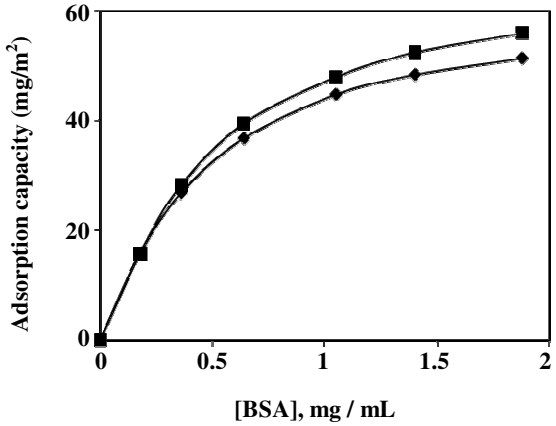

Fig. 2: Adsorption capacity of BSA onto the HA at two temperatures: ♦ 291.7 K and ■ 303.2 K.

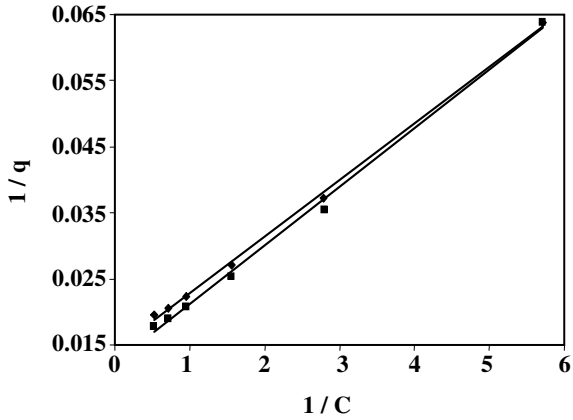

Fig. 3: The plot of 1/q versus 1/C according to Eq. (1). ♦ 291.7 K; ■ 303.2 K and lines are the best linear fitting.

The constants 1/n and K_f can be determined respectively from the slope and intercept of the linear plotting of $\ln q_e$ versus $\ln C_e$. The calculated values of K_f, 1/n and R^2 for the Freundlich model are given in Table 3.

Fig. 4 shows the experimental (points) and correlated results (lines) using Langmuir isotherm equation for adsorption of BSA onto HA as function of pH. This figure shows that the highest BSA adsorption is achieved at pH=3.8 and the lowest BSA adsorption is at pH=8. A number of effective factors in determining the amount of BSA adsorption onto the surfaces of HA are BSA charge and HA surface area and surface energy [9, 35].

The effect of pH on adsorption of BSA onto HA as presented in Fig. 4 may be interpreted with decreasing the surface energy of HA by adsorption of OH^- onto the HA at high pH. Therefore, the interaction energy of HA with BSA is reduced. Moreover, it has been found that HA possesses two different binding sites, called C and P site in the crystal surface [36]. It contains multiple-site binding character for proteins. The C sites are positively charged and thus bind to acidic groups of proteins, but the P sites lack positive charge and therefore attach to the basic groups of proteins [20, 21].

The influence of pH of solution on adsorption isotherm (296.2 K) of BSA onto HA is shown in Fig. 5. In this figure the fixed concentration of BSA is 1 mg/mL and as it is shown on decreasing pH of solution an increment in the adsorption of BSA onto HA is observed.

As BSA is a negative charge protein with $-COO^-$ groups it is expected that BSA mostly adsorbed through C site but, in solution with high pH, this site is captured with OH^- ions and as a result, the amount of BSA adsorption is decreased, whereas, the decrease of OH^- ions (pH 3.8) causes an increment in the BSA adsorption. This increase in the protein BSA adsorption is due to conformational change of adsorbed BSA onto HA [24, 35].

The conformational change of the adsorbed protein is responsible for irreversibly of protein adsorption [14, 24, 26]. When, pH of solution is high, the adsorption of BSA is reversible, whereas, it is irreversible for low values of pH.

Our results suggest that conformational change of adsorbed protein occurs only at low pH.

Also, it is known that the isoelectric point of BSA is 4.7-4.9 and is negatively charged protein. At high values of pH, the negative charge of BSA is increased and caused an increment in the electrostatic repulsion forces

Table 3: Freundlich parameters calculated, Eq.(3), from experimental results for adsorption of BSA onto HA at different pHs.

pH	K_f	1 / N	R^2
3.8	12.66	0.42	0.980
4.6	9.75	0.49	0.989
5.0	7.05	0.49	0.998
5.6	5.68	0.50	0.996
7.2	3.46	0.51	0.990
8.0	1.30	0.57	0.991

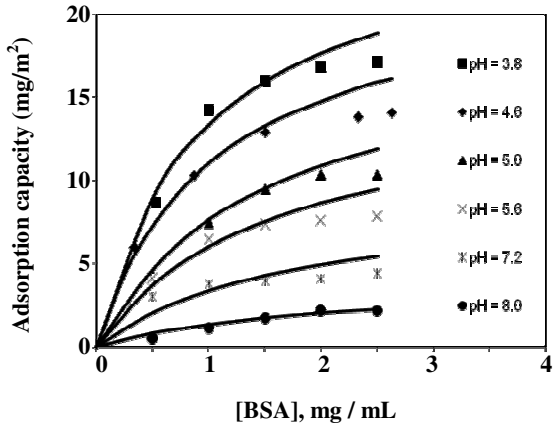

Fig. 4: The experimental (points) and correlated (lines) adsorption capacity of BSA onto the HA at different pHs and at 296.2 K.

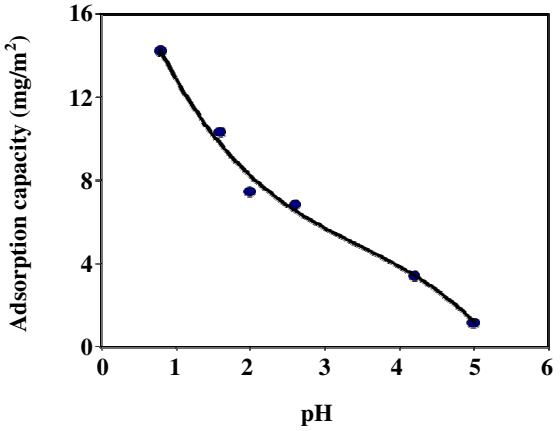

Fig. 5: The influence of pH of solution on adsorption isotherm (296.2 K) of BSA onto HA.

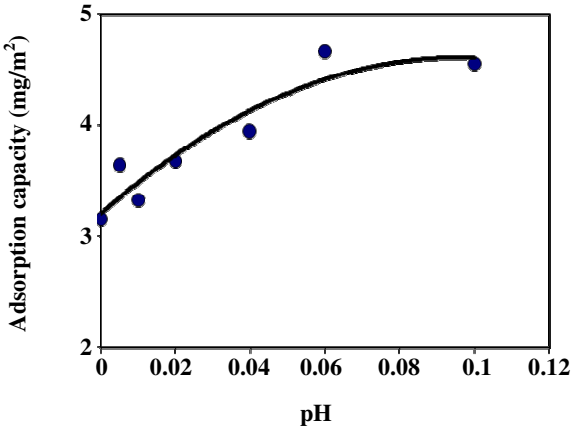

Fig. 6: Adsorption capacity of BSA onto the HA as a function of KCl concentration at 296.2 K.

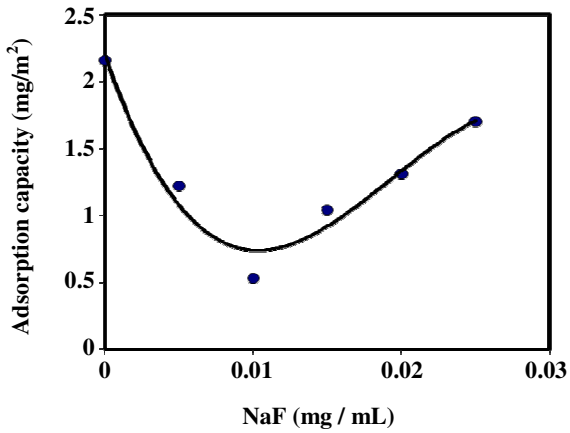

Fig. 7: Adsorption capacity of BSA onto the HA as a function of NaF concentration at 298.2 K.

between HA and BSA [37]. Consequently, the adsorption of BSA onto HA is decreased. Surface energy of adsorbent is another important factor that affects BSA adsorption. When the surface energy of adsorbent HA is increased, it causes an increment in the protein adsorption onto the substrate. Whereas, the decrease in surface energy of substrate causes stability in substrate

and the energy of interaction between substrate and surrounding liquid is decreased. Consequently, adsorption of BSA onto HA is decreased [10]. The influence of K^+ on adsorption isotherm of BSA onto HA buffered with pH 7.4 and 0.002 M KH_2PO_4/NaOH is shown in Fig. 6. Here concentration of BSA is 0.5 mg/mL. This figure shows that K^+ concentration initially causes an increase in BSA adsorption, and higher K^+ concentrations cause a decrease in the adsorption of BSA.

It is worth mentioning that potassium ions adsorb onto HA surface and decrease the negative charge of HA surface. Therefore, an enhancement of the electrostatic attraction force between HA and BSA and BSA adsorption onto HA is observed. Also from Fig. 5 the decrease in adsorption of BSA with K^+ concentration can be explained by adsorption of K^+ onto HA and the decrease of surface energy of HA due to interaction with the adsorbed K^+ onto the HA surface molecules tends to decrease the interaction of HA with the molecules in the surrounding liquid. Moreover the K^+ ions attach to P sites of HA and these ions compete with $-NH_2$ groups of amino acids of BSA for adsorption onto HA.

Our observations indicate a decrease in BSA adsorption due to this competition and the surface energy decrease predominates the initial increase due to the negative charge of HA surface.

The effect of NaF concentration on BSA adsorption onto HA buffered with pH 7.4 and 0.002 M KH_2PO_4/NaOH is shown in Fig. 7. It is known from this Figure that F^- has two effects on BSA adsorption onto HA, that is up to 0.01mg/mL of F^- BSA adsorption is decreased and then it is increased. In this case, F^- in solution is adsorbed onto HA surfaces and the negative charge of HA surface is increased, as a result, BSA adsorption onto HA is decreased due to the electrostatic repulsion forces between HA and BSA. On higher F^- concentration the surface energy of HA and consequently, the interaction with BSA is increased. The increase in surface energy predominate the effect of the electrostatic repulsion forces between HA and BSA and more BSA is adsorbed in response to this increase of HA surface energy.

The adsorption of BSA onto HA as function of phosphate buffered with pH 7.4 and 0.002 M KH_2PO_4/NaOH is shown in Fig. 8. This Figure shows that the BSA adsorption onto HA in presence of phosphate is decreased. An explanation for this behaviour is that, due to higher tendency of phosphate ions for adsorption onto HA than BSA that is the decrease in surface energy due to more phosphate ions adsorption compared with that of protein adsorption.

CONCLUSIONS

The effective factors in determining the amount of BSA adsorption onto surfaces of HA are studied. These factors are the size and charge of the protein and the

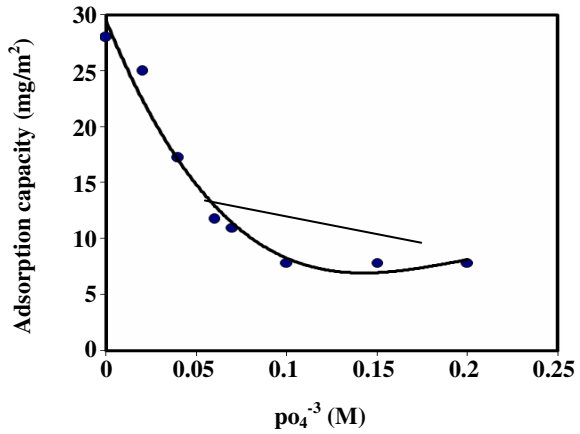

Fig. 8: Adsorption capacity of BSA onto the HA as a function of phosphate ion concentration at 297.2 K.

surface energy of HA. The electrostatic repulsion forces between HA and BSA cause a decrease in BSA adsorption onto HA. Also the BSA adsorption causes a decrease in surface energy of BSA. The effect of pH on adsorption of BSA onto HA is interpreted in terms of decreasing the surface energy of HA by adsorption of OH^- onto the HA at high pH. The results obtained in this work are treated by Langmuir isotherm equation, despite the fact that the assumptions involved in Langmuir isotherms are seldom satisfied in the case of protein adsorption [11], however, this equation provides a good fit to our results and indicates that BSA adsorption increases when the pH of the solution decreases. The effect of salts KCl and NaF and phosphate ion on BSA adsorption onto HA has also been investigated. The adsorption isotherms of BSA onto the HA surface at 291.7 K and 303.2 K were satisfactorily presented by Langmuir equation, and the maximum amount of BSA adsorption was determined.

APPENDIX A

BET equation is expressed in the following form:

$$\frac{1}{v[(P_0/P)-1]} = \frac{1}{v_m c}(P/P_0) + \frac{1}{v_m} \qquad \text{(A-1)}$$

where P and P_0 are the equilibrium and the saturation pressure of adsorbates at the temperature of adsorption, v is the volume of gas adsorbed and v_m is the the volume of gas adsorbed at monolayer coverage. c is the BET constant, which can be expressed by:

$$c = \exp[(E - E_v)/RT] \qquad \text{(A-2)}$$

Where E is the heat of adsorption for the first layer, and E_v is that for the second and higher layers and is equal to the heat of liquefaction.

According Eq. (A-1), by using the obtained experimental results, an isotherm can be plotted as a straight line with $1 / v[(P_0 / P) - 1]$ versus P / P_0 in the range of $0.05 < P / P_0 < 0.35$. The value of the slope and the y-intercept of the line are used to calculate the volume of gas adsorbed at monolayer coverage v_m and the BET constant c.

A total surface area S_t and a specific surface area S are evaluated by the following equations:

$$S_t = (v_m N s)/M \qquad \text{(A-3)}$$

$$S = S_t / a \qquad \text{(A-4)}$$

Where N, s, M and a are respectively Avogadro's number, adsorption cross section, molecular weight of adsorbate and weight of sample solid.

NOMENCLATURE

a	Weight of sample solid
BSA	Bovine Serum Albumin
c	The BET constant
C	Equilibrium solution concentration of solute, mg /unit solution
E_1	The heat of adsorption for the first layer
E_L	The heat of adsorption for the second and higher layers
HA	Calcium Hydroxyl Apatite
K_a	Langmuir equilibrium constant unit solution/mg of adsorbed solute
M	Molal concentration
M	Molecular weight of adsorbate
N	Avogadro's number
P	The equilibrium pressure of adsorbate at the temperature of adsorption
P_0	The saturation pressure of adsorbate at the temperature of adsorption
q	Equilibrium concentration of adsorbed solute mg /unit adsorbent
q_s	Maximum concentration of adsorbed solute mg /unit adsorbent
R	Gas constant
s	Adsorption cross section
S	Specific surface area

S_t	Total surface area
T	Temperature
v	The adsorbed gas volume
v_m	The monolayer adsorbed gas volume

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